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Analytical Approaches to the Study of the Disposition of Myelotoxic Agents

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A correlation of myelotoxic effect with concentration or a foreign compound of its metabolite at the site of action may provide useful insights into the mechanism of toxic action and/or its amelioration. This correlation requires sensitive and specific assay methods. This communication describes useful methods for the study of benzene disposition in rodents. The assays are sensitive, specific, and rapid. They rely on gas chromatography-mass spectrometry and on high performance liquid chromatography. These methods have allowed measurement of catechol, phenol, and hydroquinone in samples of rodent bone marrow following inhalation exposure to benzene. Their application to the study of benzene metabolism in rat bone marrow *in situ* is also described.

Introduction

The study of myelotoxicity, like the study of other forms toxicity, requires a thorough knowledge of the disposition of the toxicant under investigation. The ultimate goal is the identification of the proximal toxicant and a description of the interactions of that toxicant with cellular constituents of the target issue. Properly designed disposition studies can yield useful information on the structure and concentration of putative proximal toxicants at the site of action. In this respect, disposition studies of myelotoxic agents are similar to disposition studies of any other type of toxic agent.

Analysis for myelotoxic agents and their metabolites in the target tissue does, however, present some interesting problems. The scarcity of bone marrow tissue in rodents requires extremely efficient and sensitive methods for analysis. The studies described here were done with benzene. In addition to problems associated with sampling and analysis of small amounts of tissue, benzene and its metabolites pose analytical problems because of their volatility, instability, and/or highly polar nature. Successful circumvention of some of these problems has been accomplished with combined gas chromatography-mass spectrometry and high performance liquid chromatography. We have used these techniques to describe the pharmacokinetics of benzene after inhalation exposure in rats (*1*) to demonstrate that bone marrow has metabolic activity toward benzene (*2*), and to begin to correlate benzene metabolism *in vitro* with alterations in *in vivo* benzene toxicity produced by pretreatment with other foreign compounds (*3*).

Methods and Results

Male, Fischer-344 rats (200-250 g) were exposed to 500 ppm benzene in a 1100 liter dynamic air flow chamber for 6 hr. The time required for benzene concentration to reach 99% of the target concentration was 20.3 min. At the end of the exposure period the animals were removed. Groups of three were sacrificed by decapitation 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, and 9 hr after removal. Heparinized blood, perirenal fat, and bone marrow were analyzed for benzene. The goals in developing a method for benzene in these tissues included sufficient sensitivity to measure benzene in bone marrow, specificity sufficient to ensure that endogenous compounds did not interfere with the measurement, and a minimi-

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zation of sample preparation so that the volatility of benzene would not lead to erroneously low values.

In order to correct for the volatility of benzene, deuterated (d_6) benzene (Merck and Co., Inc., Quebec, Canada) was added as an internal standard immediately after removal of the tissues. Methanol (0.1 ml) containing 1 μg benzene-d_6/ml was added to 0.1 ml blood. A 15 to 25 mg portion of bone marrow was mixed with 0.1 ml methanol containing 1 μg benzene-d_6/ml. All other tissues were homogenized on ice in 3 ml methanol (containing 1 μg benzene-d_6/ml)/g of wet weight tissue. All samples were sonicated and mixed then centrifuged at 1500*g* for 15 min. Then 1-5 μl of the supernate was injected onto the gas chromatograph-mass spectrometer (Finnegan 4021, Finnegan Corp., Sunnyvale, Calif.). The mass spectrometer was operated in the selected ion monitoring mode and alternately focused on *m/e* 78 (parent ion of benzene) and *m/e* 84 (parent ion of benzene-d_6). Reconstructed mass chromatograms of a typical standard are shown in Figure 1. The gas chromatography column was Tenax GC (Suppleco, Inc., Bellafonte, Pa.) packed in a 2 m × 2 mm (i.d.) glass column. Helium carrier gas was admitted to the column at 20 ml/min. The column was temperature programmed from 190 to 225°C at a rate of 24°C/min. The ratio of the area under the peak at *m/e* 78 to the area under the peak at *m/e* 84 was plotted against the concentration of benzene in the standards (Fig. 2). The standard curve was linear over final benzene concentrations of 100 ng/ml to 100 μg/ml. Thus, the method allowed quantitation of benzene at concentrations between 200 ng/ml and 200 μg/ml in blood, between 200 ng/g and 200 μg/g in bone marrow (if volumes injected onto gas chromatography-mass spectrometer were increased to 5 μl), and between 400 ng/g and 400 μg/g in other tissues. Disappearance of benzene from blood, bone marrow, and perirenal fat is shown in Figure 3.

Three metabolites of benzene, phenol, catechol, and hydroquinone, were measured in blood and bone marrow from the above rats. A 5-ml portion of methanol was added to 1.0 ml blood or 15-25 mg bone marrow. Methanol (0.1 ml) containing 1.0 μg phenol-d_6/ml (Merck & Co., Quebec, Canada) was added to the mixture which was then mixed vigorously and centrifuged at 1500*g* for 15 min. The supernate was transfered to a clean tube and 0.1 ml of 0.1*M* KOH was added. The samples were evaporated to dryness under a stream of N_2. Trifluoroacetic anhydride (Aldrich Chemical Co., Milwaukee, Wisc.) (500-100 μl) was added, and aliquots of 1-2 μl were injected onto the gas chromatograph-mass spectrometer. The resulting trifluoroacetate derivatives were separated on a 2 m × 2.0 mm i.d. glass column

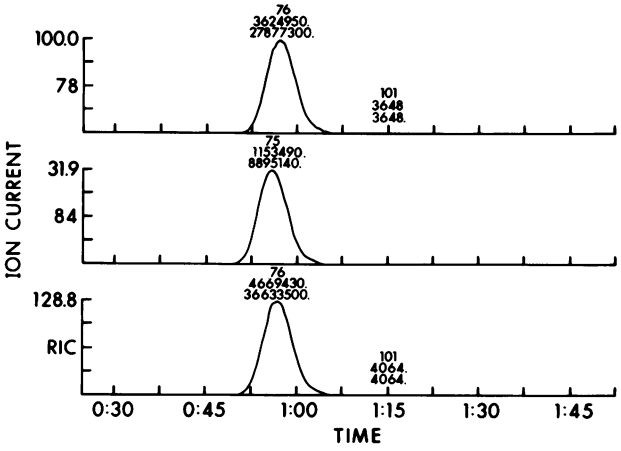

FIGURE 1. Ion chromatograms of benzene and benzene-d_6: (top) chromatogram for benzene, *m/e* 78 was monitored; (middle) chromatogram of benzene-d_6 for which *m/e* 84 was monitored; (bottom) sum of the other two chromatograms. Numbers above the peaks are, from top to bottom, scan number, height, and area.

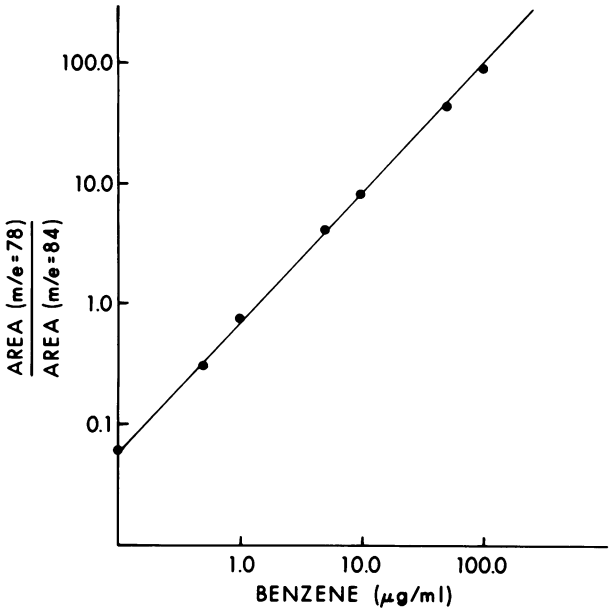

FIGURE 2. Typical standard curve for benzene plotted on a log-log scale.

packed with 3% OV-1 on Chromasorb W (Supelco, Inc., Bellefonte, Pa.) and held at 90°C. The carrier gas was helium at a flow rate of 20 ml/min. The ions monitored by the mass spectrometer were *m/e* 195, the parent ion for fluoroacetylated phenol-d_5 (one deuterium atom is lost in the derivatization reaction); *m/e* 190, the parent ion for fluoroacetylated phenol and *m/e* 302, the parent ion for fluoroacetylated catechol and hydroquinone. A typical mass chromatogram for a standard mixture is shown in Figure 4.

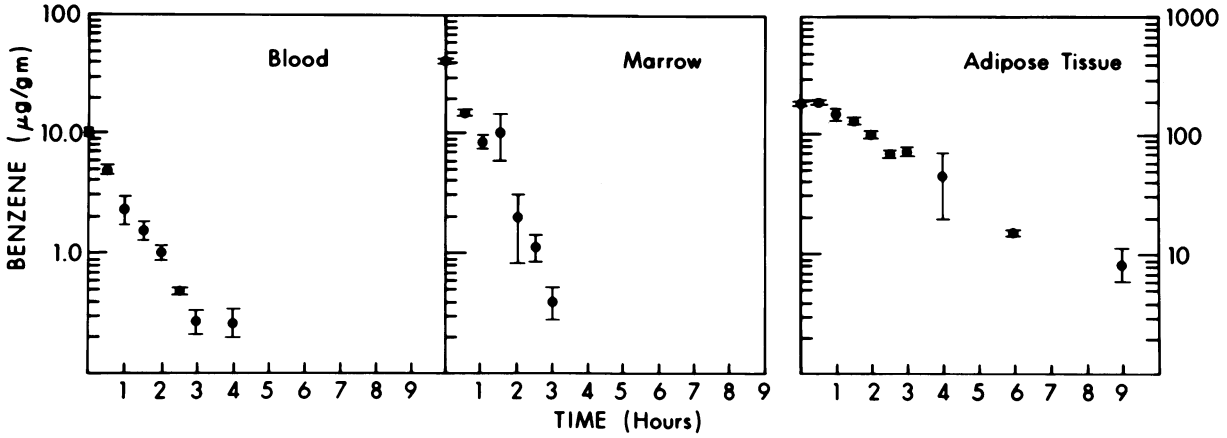

FIGURE 3. Disappearance of benzene from blood, bone marrow, and adipose tissue following 6 hr exposure to 500 ppm benzene in air.

A standard curve was prepared by carrying 500 pg to 10 ng of phenol, catechol, or hydroquinone through the above procedure. A standard curve is given in Figure 5. Sensitivities were 50 ng of metabolite/g bone marrow or 500 pg of metabolite/ml blood. Concentrations of phenol, catechol, and hydroquinone at several times following the end of exposure and given in Table 1.

Both normal-phase and reversed-phase high performance liquid chromatography have been successfully applied to studies of benzene metabolism. There is evidence in the literature that rat bone marrow is metabolically competent toward xenobiotics (*4*), but there has only recently been a demonstration of bone marrow's ability to metabolize benzene *in situ* (*2*). Because certain benzene metabolites, namely, sulfate and glucuronide conjugates, are not readily analyzed by combined gas chromatography-mass spectrometry, we employed normal-phase

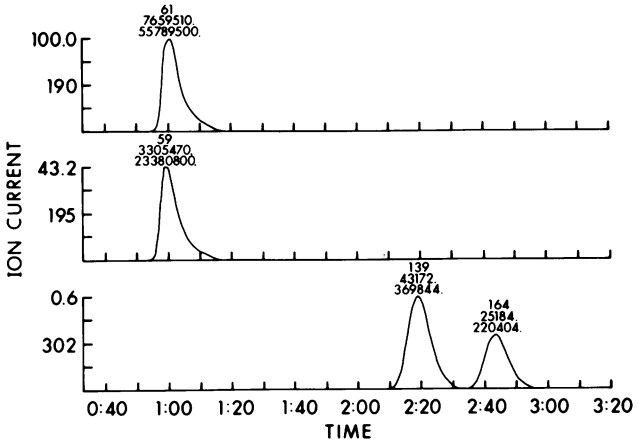

FIGURE 4. Ion chromatograms of (top) fluoroacetylated phenol, (middle) phenol-d_5, (bottom, first peak) catechol and (bottom, second peak) hydroquinone. Numbers above peaks as in Figure 1.

Table 1. Concentration of phenol, catechol, and hydroquinone in rat blood and bone marrow after a 6 hr exposure to 500 ppm benzene in air.

Time from end of exposure, hr	Phenol[a]		Catechol[a]		Hydroquinone[a]	
	Blood, ng/ml	Bone marrow, ng/g	Blood, ng/ml	Bone marrow, ng/g	Blood, ng/ml	Bone marrow, ng/g
0.5	1958 ± 540	6101 ± 1500	53 ± 23	7215 ± 704	18 ± 8	58480 ± 3037
1.0	1108 ± 236	2808 ± 1600	14 ± 6	5097 ± 1586	59 ± 11	15010 ± 4320
1.5	1431 ± 521	19410 ± 9517	30 ± 12	6982 ± 3633	50 ± 20	19150 ± 1070
2.0	444 ± 64	ND	59 ± 10	5033 ± 1027	89 ± 20	14030 ± 2790
2.5	292 ± 35	ND	72 ± 10	3476 ± 266	253 ± 100	9545 ± 811
3.0	243 ± 61	1918 ± 1565	61 ± 30	7223 ± 1725	68 ± 9	26630 ± 5911
4.0	197 ± 76	894 ± 730	ND	5393 ± 1115	102 ± 35	42200 ± 18130
6.0	62 ± 25	ND	37 ± 12	2614 ± 850	52 ± 20	7050 ± 2150
9.0	ND	ND	19 ± 12	13160 ± 4036	66 ± 30	35390 ± 1042

[a]Values are means of 3-6 rats ± S.E.M.

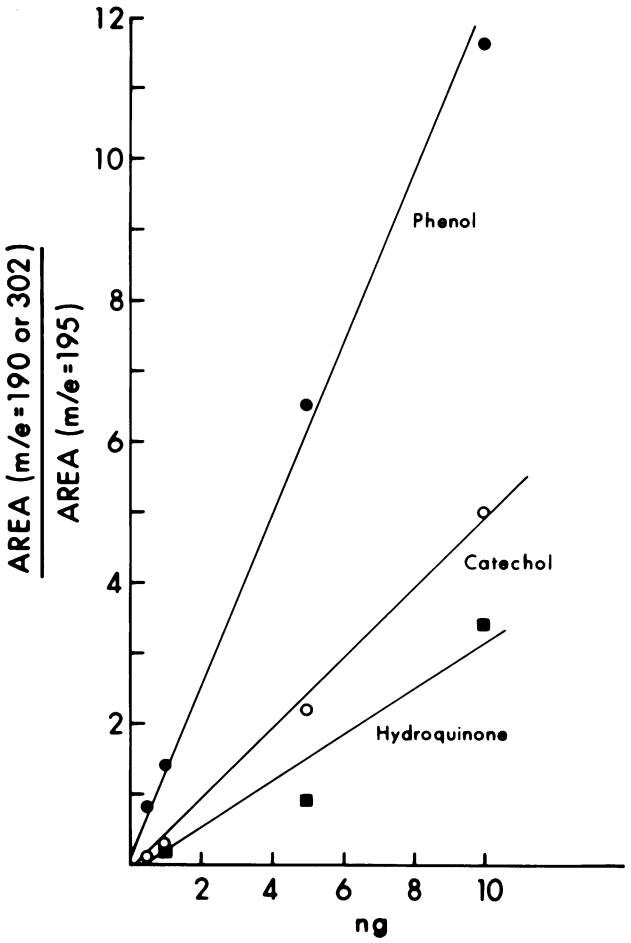

FIGURE 5. Typical standard curves for phenol, catechol, and hydroquinone.

high performance liquid chromatography. Male, Fischer-344 rats were aesthetized with an anileridine-ketamine mixture; the left common iliac vein and artery were cannulated and the isolated hind limb was perfused with citrated, oxygenated rat blood at a flow of 1 ml/min. Between 0.5 and 2.0 mCi of ^{14}C-benzene (39 mCi/mmole) was introduced directly into the marrow via a hole drilled at the distal end of the femur. Bone wax was used to close the hole, and blood was collected from the iliac vein for 1 hr in 10-min fractions. The bone marrow was removed at the end of the hour perfusion, and blood and bone marrow were analyzed for benzene metabolites. Blood (10 ml) or bone marrow (15-25 mg) homogenized in 1.0 ml phosphate buffer, pH 7.4, was mixed with 50 μg each phenol, catechol, hydroquinone, and 1,2,4-benzenetriol. The pH was adjusted to 1.0 with 1.0*M* HCl, and the mixture was extracted three times with two volumes of ethyl acetate. The ethyl acetate layers were combined, dried over anhydrous magnesium sulfate, and filtered. After evaporation under a stream of N_2 to a final volume of about 1.0 ml, the ethyl acetate fractions were passed over a short silica gel column (Waters Associates, Milford, Mass.). The sample was then further concentrated to ~ 20 μl and then injected onto the high-pressure liquid chromatograph (Waters Associates, Milford, Mass.). Separation was accomplished with two DuPont Zorbax silica columns. The flow was programmed from 0.8 ml/min to 1.6 ml/min over 20 min. The solvent system used was 74.9% methylene chloride, 25% ethyl acetate, 0.1% methanol, with 100 μl of 88% formic acid added per 100 ml solvent. A typical elution profile is shown in Figure 6. Radioactivity co-eluted with phenol, catechol and hydroquinone. Concentrations of these compounds in blood and bone marrow from *in situ* perfusions are given in Table 2.

The normal-phase separation of benzene metabolites was satisfactory, but the time-consuming and complicated sample preparation limited its utility in more extensive studies with more samples and lower amounts of radioactivity. We have now developed a reversed-phase high-performance liquid chromatographic procedure which allows samples to be injected in aqueous systems, thereby reducing sample handling and shortening analysis time. Trichloroacetic acid (125 μl, 15% *w/v*) was added to an incubation mixture (0.9 ml). The

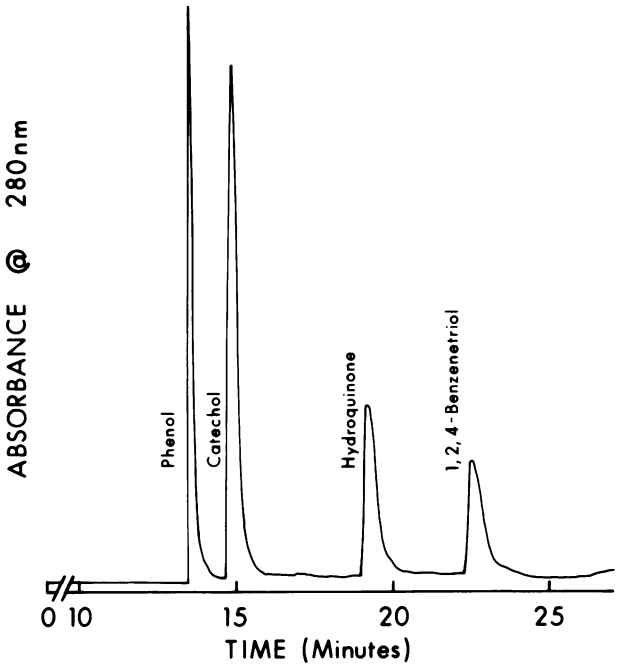

FIGURE 6. Normal-phase high pressure liquid chromatogram of benzene metabolites. See text for conditions.

Table 2. Concentrations of phenol, catechol, and hydroquinone in venous blood of isolated hind limb perfusion after injection of benzene into bone marrow *in situ*.

Collection period, min	Phenol, pmole/10 min[a]	Catechol, pmole/10 min[a]	Hydroquinone, pmole/10 min[a]
0-10	4540 ± 773	222 ± 74	93 ± 7
10-20	664 ± 151	59 ± 22	11 ± 5
20-30	247 ± 41	184 ± 117	4 ± 3
30-40	221 ± 29	49 ± 20	11 ± 8
40-50	192 ± 123	68 ± 27	12 ± 10
50-60	59 ± 28	21 ± 9	11 ± 7

[a]Values are means for 3-4 preparations ± S.E.M.

resulting mixture was centrifuged at 2000*g* for 5 min, and 100-200 µl of the supernate was injected onto the high-pressure liquid chromatograph. The column used was octadecylsilane (Waters Radial Pak A) or octylsilane (Merck RP-8). Compounds were eluted in 20 min by a linear gradient from 100% water containing 100 µl formic acid/l. to 100% methanol containing 100 µl formic acid/l. A typical elution profile of benzene metabolites is shown in Figure 7.

A first application of the new analytical procedure has been the study of benzene metabolism *in vitro* by various tissues. When hepatic 10,000*g* supernate was incubated in the presence of ^{14}C-benzene and an NADPH regenerating system, two metabolite peaks appeared in the high performance liquid chromatogram. These peaks co-eluted with hydroquinone and phenol. When the same incubation was repeated with the addition of ATP, sodium sulfate, and uridine diphosphoglucuronic acid, the peaks co-eluting with hydroquinone and phenol decreased in size. An early-eluting peak, presumably a conjugate, appeared. Work to characterize fully the metabolites formed from benzene *in vitro* is continuing.

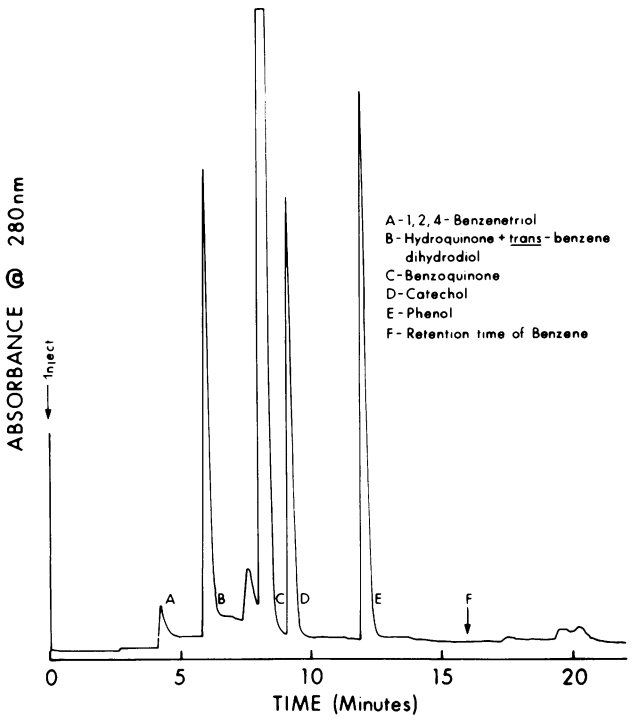

FIGURE 7. Reversed-phase high pressure liquid chromatogram of benzene metabolites. See text for conditions.

Discussion

Several methods for studying the disposition of benzene have been described. Each has its particular advantages and disadvantages, and each could be modified to study the disposition of other myelotoxic agents as well. The mass spectrometric methods described have the disadvantage of requiring expensive equipment and trained operators, but their sensitivity, specificity and simplicity of sample preparation make them very attractive. Using these methods we were able to show that half-lives for bezene in all tissues studied (except perirenal fat) were similar and that benzene is concentrated in bone marrow and fat as well as in organs of metabolism and excretion, the liver and kidney. In addition, concentration of phenol in blood and bone marrow declined rapidly after termination of exposure, but catechol and hydroquinone did not. This suggests a possibility of accumulation of these two potentially myelotoxic metabolites.

While our gas chromatographic-mass spectrometric methods required that we decide in advance which compounds to quantitate, no such requirement was imposed by the high performance liquid chromatographic procedures employing radiolabelled benzene. These techniques allowed us to demonstrate the metabolism of benzene by bone marrow *in situ* and to study the *in vitro* metabolism of benzene, including conjugate formation.

As this publication is intended to be a description of methods available for the study of xenobiotic disposition, only brief examples of the data obtained

have been given. A fuller presentation and discussion of the disposition data can be found in the cited publications.

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